OXALIC ACID PRODUCTION BY SPECIES OF ARMILLARIA IN THE BOREAL MIXEDWOOD FOREST

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SUMMARY

Oxalic acid, in its free and bound forms, was produced by the four biological species of *Armillaria* (I: *A. obscura*, III, V, VII: *A. bulbosa*) that are indigenous to the Boreal Mixedwood Forest of Ontario. Two crystal forms were observed by scanning electron microscopy: one was found on the surface of the medium and was prismatic in shape, whereas the other was found embedded in the medium and was saucer shaped. Both forms were stained by silver nitrate-hydrogen peroxide and silver nitrate-dithiooxamide. X-ray dispersive analysis revealed the presence of only one cation: calcium. Oxalic acid was detected in the culture filtrates of all biological species of *Armillaria* by gas liquid chromatography. Variations in production of oxalic acid among isolates of biological species I and biological species V were observed.

INTRODUCTION

Oxalic acid is produced by many plant-pathogenic fungi. Bateman and Beer (1965) postulated that oxalic acid lowered the pH of the infected tissues, which in turn made them more susceptible to destruction by polygalacturonase. Kritzman et al. (1977) concluded that oxalic acid was an essential factor in the pathogenicity of *Sclerotium rolfsii* on lima bean seedlings.

Wood-decay fungi have been shown to produce oxalates *in vitro* (Hütterman et al. 1980, Holdenrieder 1982, Arnott and Webb 1983). Hamada (1940) reported that *Armillaria mellea* produced oxalic acid in culture. Mallett and Colotelo (1984) did not observe this acid in the exudates from the rhizomorphs of *A. mellea*. In the present study, chemical tests and scanning electron microscopy were used to demonstrate the production of oxalic acid, in its pure and chelated form, by the four species of *Armillaria* present in the Boreal Mixedwood Forest of Ontario.

MATERIAL AND METHODS

Isolates of the four biological species (I, III, V, VII) of Armillaria found in the Boreal Mixedwood Forest (Dumas 1988) were used. Armillaria was cultured on a medium consisting of 4.0 g L-asparagine, 1.75 g KH_2PO_4, 0.75 g $MgSO_4$, 1.0 mg thiamin.HCl, 20.0 mg O-aminobenzoic acid, 15.0 g Bacto-agar and 20.0 g glucose per L of distilled water (Garraway 1975). The pH was adjusted to 6.0 prior to sterilization at 121°C for 20 minutes. The glucose was autoclaved separately, and O-aminobenzoic acid was prepared in 50% ethanol and sterilized by filtration through a 0.2-μm filter. The final volume of ethanol per L of medium was 2 ml. Each 100 x 15 mm petri dish received 25 ml of culture medium and was inoculated with 3- to 4-mm plugs of inoculum. The plates were sealed with parafilm and incubated at 25°C in the dark.

To observe the crystals with a scanning electron microscope, four-week-old cultures were used. Sections were cut from air-dried, unfixed cultures and attached to aluminum stubs with double-coated sticky tape. Specimens were coated with carbon and examined with a Cambridge microscope at 25 kV.

The production of oxalic acid by six isolates of biological species I, by two isolates of biological species V and by one isolate each of biological species III and VII, was monitored weekly with the aid of Garraway's (1975) liquid medium, as follows: 50 ml of medium were dispensed into 250-ml flasks stoppered with cotton plugs; 15 plugs (each 8 mm in size) were cut from a four-week-old culture, grown on the same medium, and added to 15 ml of sterile distilled water; the mixtures were then homogenized with a Sorvall Omnimixer at a setting of 5 for three minutes.Each flask received 1 ml of the stirring slurry and was incubated at 25°C in the dark.

Mycelia were collected by vacuum filtration on pre-weighed Whatman #4 filter papers. They were dried for 24 hours at 70°C and stored over $CaSO_4$ until a constant weight was achieved; 5 ml of culture filtrate, in 100 x 13 mm tubes, were frozen overnight at -40°C and were subsequently lyophilized for 24 hours. The dried samples were methylated with 30% sulfuric acid in methanol according to the procedure of Sutikno et al. (1987). The amount of oxalate was determined on a Hewlett-Packard 5830 gas chromatograph equipped with a flame ionization detector. The glass liquid column (1.82 m x 2.0 mm I.D.) was packed with 10% Fluororad FC 431 + 1% H_3PO_4 on Chromosorb W-HP 80/100 mesh. The injector and detector temperatures were kept constant at 100°C and 250°C, respectively. The column temperature was maintained at 125°C for three minutes and programmed to increase at a rate of 30°C/min to a final temperature of 160°C and held at this temperature for two minutes. The gas flow rates were: nitrogen 30 ml/min, hydrogen 60 ml/min, and air 240 ml/min. Sample size was 2 μL. The amount of oxalate was calculated with a Hewlett Packard 18850A terminal and oxalic acid as an external standard.

RESULTS AND DISCUSSION

Crystals of oxalic acid were produced by the four biological species of Armillaria and were found interspersed with the mycelia (Fig. 1) and on the surface of the medium. Scanning electron microscopy revealed the presence of two crystal shapes. One was prismatic, and was always found on the surface of the medium (Fig. 2). This form is indicative of Weddellite (Frey-Wyssling 1981). The other crystal configuration was found within the agar layer and could be seen only after the overlying layer of medium was burned away by the x-ray beam. These crystals were all saucer shaped (Fig. 3). Both crystal forms were insoluble in 5% acetic acid but soluble in 0.5 N hydrochloric acid, which indicated that they were not composed of calcium phosphate or carbonate (Yasue 1969, Franceschi and Horner 1980). The crystals were stained black by silver nitrate-hydrogen peroxide (Pizzolato 1964) and silver nitrate-dithiooxamide (Yasue 1969), which are specific stains for calcium oxalate. Energy-dispersive x-ray analysis of the two crystal forms demonstrated that calcium was the sole cation (Fig. 4). The presence of oxalic acid in the culture filtrates of the four biological species of Armillaria was confirmed by the titration method of Kritzman et al. (1977), and by gas liquid chromatography according to the method of Sutikno et al. (1987).

To determine if oxalic acid was produced, gas liquid chromatography was used. Purified oxalic acid and calcium oxalate were added to liquid Garraway's medium in order to determine the best esterification agent. These agents included 14% boron trifluoride in methanol, by the procedure of Calaf et al. (1982), N,O-Bis (Trimethylsilyl) trifluoroacetamide (BSTFA) (Nicolai and Zilliken 1974), Tri Sil-BSA Formula P (Tanaka and Hine 1982) and various concentrations of sulfuric acid in methanol (Sutikno et al. 1987). The most efficient esterification agent was 30% methanol in sulfuric acid.

The retention times of the oxalic acid produced by Armillaria and of a laboratory sample were the same. Extraneous peaks in the chromatogram were from the medium (Fig. 5). When the culture filtrates were spiked with oxalic acid or calcium oxalate the retention time remained the same but the concentration changed, and this further confirmed its identification.

The minimum detectable amount that could be accurately determined was 25 µg/ml. For the majority of isolates this amount was not detectable in the first week of growth and therefore a lag period of one week was established before sampling. The isolates of biological species I were variable in their production of oxalic acid. Isolates Ia, Ib, Ie, and If did not produce appreciable amounts of oxalic acid in the first sampling period (two weeks of growth), whereas isolates Ic and Id produced amounts that were easily detected. Production of oxalic acid over the following two sample periods remained relatively constant;

however, during the fourth sampling period (five weeks of growth), the amount of oxalic acid produced by Ia, Ic, and Ie increased whereas the amounts produced in the culture filtrates of Ib, Id, and If decreased (Table 1).

A much larger variation in production of oxalic acid was noticed with biological species V. Isolate Va produced a maximum amount of 53.1 μg of oxalic acid per mg dry weight of mycelium. Isolate Vb, however, produced a maximum amount of 391.2 μg/mg during the third sampling period and, like some isolates of biological species I, the amount decreased during the final sampling period (Table 1). Biological species III and VII produced relatively large amounts of oxalic acid; the maximum production occurred during the third sample period, with a subsequent decrease during the final period (Table 1).

Table 1. Production of oxalic acid (μg/mg dry weight of mycelium) by the species of Armillaria present in the Boreal Mixedwood Forest of Ontario.

Isolate	Sampling Period (weeks) 2	3	4	5
Ia	77.3	129.9	101.3	145.4
Ib	40.7	133.1	91.6	83.3
Ic	148.6	156.6	154.3	273.5
Id	218.3	180.8	206.8	148.3
Ie	59.1	124.3	102.6	160.2
If	49.0	120.5	81.9	32.5
III	105.9	116.3	148.9	128.5
Va	*	40.1	33.8	53.1
Vb	56.5	212.2	391.2	328.1
VII	153.1	159.7	269.9	138.3

* undetectable

The amount of oxalic acid produced was not related to the amount of mycelium produced. It appears that production of oxalic acid is a reflection of the inherent productive ability of certain isolates. This difference may reflect variations in the degree of pathogenicity of Armillaria isolates, as evidenced by Rishbeth (1983) and Mallett and Hiratsuka (1988), which was correlated with virulence of Endothia parasitica (Havir and Anagnostakis 1983). The correlation between oxalic acid production and pathogenicity of the Armillaria isolates of the Boreal Mixedwood Forest will be investigated further.

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LITERATURE CITED

Arnott, H.J. and M.A. Webb. 1983. The structure and formation of calcium oxalate crystal deposits on the hyphae of a wood rot fungus. Scanning Electron Microsc. 4,1747-1758.

Bateman, D.F. and S.V. Beer. 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturenase during pathogenesis by *Sclerotium rolfsii*. Phytopathology 55,204-211.

Calaf, R., P. Marie, T. Ayavou and J. Reynaud. 1982. Étude critique du dosage de l'acide oxalique urinaire par chromatographie en phase gazeuse. Ann. Biol. Clin. 40,673-679.

Dumas, M.T. 1988. Biological species of *Armillaria* in the mixedwood forest of northern Ontario. Can. J. For. Res. (In press).

Franceschi, V.R. and H.T. Horner Jr. 1980. Calcium oxalate crystals in plants. Bot. Rev. 46:361-427.

Frey-Wyssling, A. 1981. Crystallography of the two hydrates of crystalline calcium oxalate in plants. Amer. J. Bot. 68, 130-141.

Garraway, M.O. 1975. Stimulation of *Armillaria mellea* growth by plant hormones in relation to the concentration and type of carbohydrates. Eur. J. For. Pathol. 5,35-43.

Hamada, M.v. 1940. Physiologisch-morphologische studien Über *Armillaria mellea* (Vahl.) Quél., mit besonderer rÜcksicht auf die oxal-saurebildung ein nachtrag zur mykorrhiza von *Galeola septentrionalis* Reichb. f. Jap. J. Bot. 10,387-464.

Havir, E.A. and S.L. Anagnostakis. 1983. Oxalate production by virulent but not by hypovirulent strains of *Endothia parasitica*. Physiol. Plant Pathol. 23,369-376.

Holdenrieder, O.V. 1982. Kristallbildung bei *Heterobasidion annosum* (Fr.) Bref. (*Fomes annosus* P. Karst.) und anderen holzbewohnenden Pilzen. Eur. J. For. Path. 12,41-58.

Hüttermann, A., W.E. Kuhl and F. Chet. 1980. The effect of L-threonine on oxalic acid synthesis by Fomes annosus. Eur. J. For. Pathol. 10,339-344.

Kritzman, G., I. Chet and Y. Henis. 1977. The role of oxalic acid in the pathogenic behavior of Sclerotium rolfsii Sacc. Exper. Mycol. 1,280-285.

Mallett, K.I. and N. Colotelo. 1984. Rhizomorph exudate of Armillaria mellea. Can. J. Microbiol. 30,1247-1252.

Mallett, K.I. and Y. Hiratsuka. 1988. Inoculation studies of lodgepole pine with Alberta isolates of the Armillaria mellea complex. Can. J. For. Res. 18,292-296.

Nicolai, H.v. and F. Zilliken. 1974. Gaschromatographische bestimmung von oxalsäure, malonsäure und bernsteinsäure aus biologischem material. J. Chromatogr. 92,431-434.

Pizzolato, P. 1964. Histochemical recognition of calcium oxalate. J. Histochem. Cytochem. 12,333-336.

Rishbeth, J. 1983. Pathogenicity tests for Armillaria. pp. 131-139 in G.A. Kile, Ed. Proc. 6th IUFRO Conf. Root and Butt Rots of Forest Trees. CSIRO, Melbourne, Austr.

Sutikno, A.I., D. Suherman and B. Tangendjaja. 1987. A screening method for total oxalate determination in plant material by gas chromatography. J. Sci. Food Agric. 39,233-238.

Tanaka, K. and D.G. Hine. 1982. Compilation of gas chromatographic retention indices of 163 metabolically important organic acids, and their use in detection of patients with organic acidurias. J. Chromatogr. 239,301-322.

Yasue, T. 1969. Histochemical identification of calcium oxalate. Acta. Histochem. Cytochem. 2,83-95.

Fig. 1. Crystals of calcium oxalate in the medium of an *Armillaria* culture.

Fig. 2. Prismatic crystal of calcium oxalate produced by *Armillaria*.

Fig. 3. Saucer-shaped crystal of calcium oxalate produced by *Armillaria*.

Fig. 4. X-ray dispersive emission spectrum of calcium oxalate crystal produced by *Armillaria*.

Fig. 5. Gas-liquid chromatograms of a) uninoculated Garraway's medium, b) oxalic acid, c) culture filtrate from *Armillaria*.

Fig. 1.

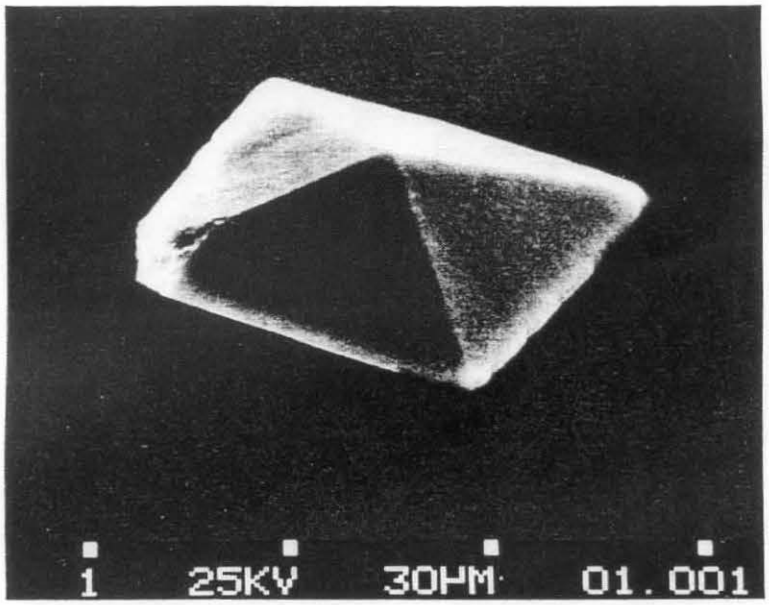

Fig. 2.

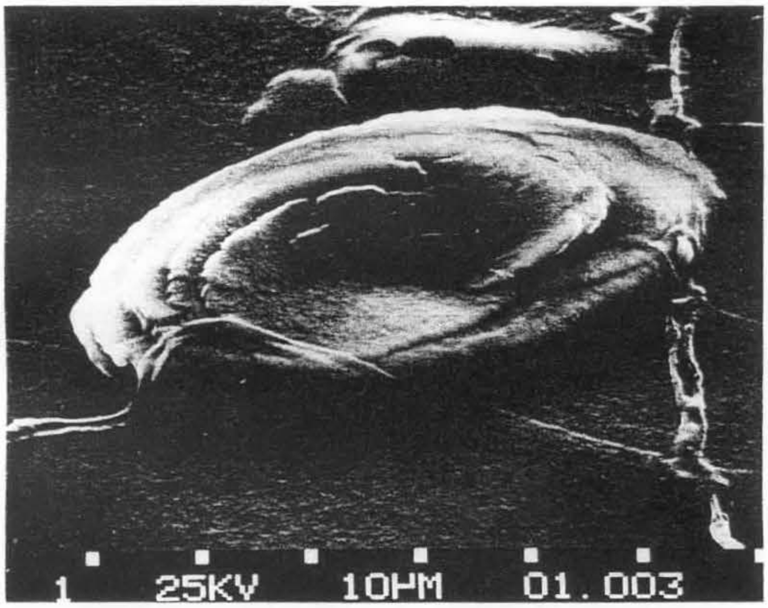

Fig. 3.

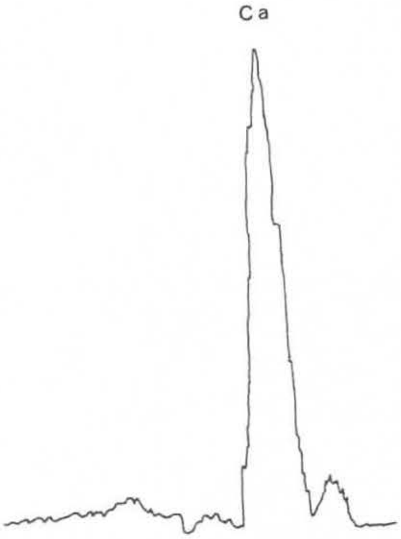

Fig. 4.

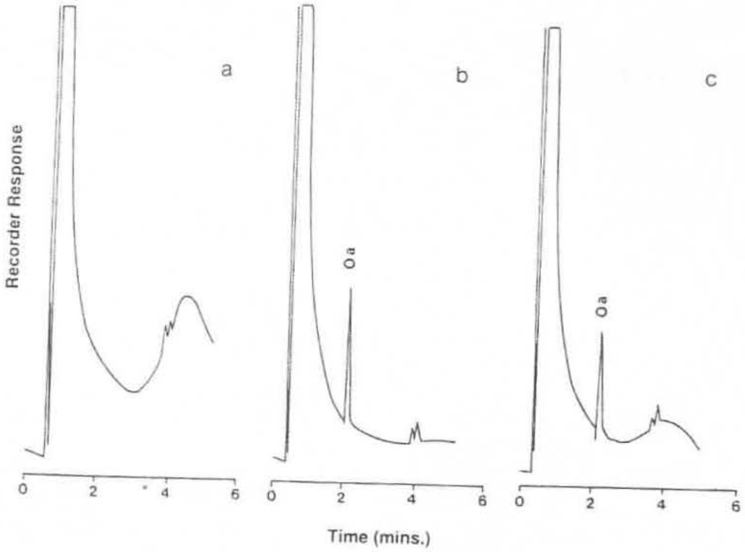

Fig. 5.